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Elucidating the mechanism of pit formation in cherries for improving storage capacity

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Project award year: 2015
Three year research project

Scientific abstract of the project

Pitting in cherry, the collapse of the fruit surface, develops during storage in perfect fruit. The overall goal of this project was to understand the underlying mechanism(s) leading to pit formation and its amelioration by ethanol and calcium. We planned to: 1. Determine calcium distribution in sensitive/resistant cultivars; 2. Compare the transcriptome of sensitive/ resistant cultivars during storage; 3. Characterize the transcriptomic response of sensitive/resistant cultivars following an impact; 4. Study the kinetics of changes in membranes, ROS, and cell viability during storage with/without an impact; 5. Follow the kinetics of physiological and molecular changes after treatments with calcium and ethanol.

We discovered that plant maturity affects pitting; mature fruit are more sensitive than less mature fruit. Although pitting develops after harvest even at 20C, in most of our storage experiments, exposure to 0C induced a more severe pitting than exposure to 4-5C. Hence, advanced maturity and low temperature enhances pitting.

Impact, either by dropping the fruit or by applying localized controlled force, increased or caused pitting, respectively. However, pitting is not caused only by an impact. Since induced pitting by applying localized force did not always reflect the natural pitting sensitivity after storage, we concluded that this method cannot be used as a phenotypic tool to predict differences between cultivars or years in sensitivity to pitting, despite publications in the literature. Nevertheless, we confirmed that generally, Lapins (Israel, California) and Brooks (California) are more sensitive than Bing (Israel, California) to the development of natural pitting.

Following apoplastic calcium in the different cultivars did not reveal consistent differences between cultivars, mainly because no difference was found in induced pitting between cultivars. Nevertheless, analysis of apoplastic calcium in the two developmental stages in harvest, revealed that the more mature stage had lower apoplastic calcium than less mature fruit. This observation may suggest that indeed, lower apoplastic calcium is related to higher sensitivity to pitting development which occurs in a more advanced stage. In conclusion, the role of low apoplastic calcium in pitting development has to be further investigated.

Transcriptome analysis of early-cold exposed cherries was performed on non-impacted Bing and Lapins in two consecutive years in Israel and on impacted Bing and Brooks fruit in California. There was a major difference in the transcriptome of cherries from California and from Israel, which coincide with a major difference in pitting sensitivity, being higher in Israel. The transcriptome analysis revealed that components know to be involved in acclimation to a cold correlated with the resistance to pitting in the two locations (Israel, California). However, the components were different in the two locations and their induction by cold during storage did not always explain the sensitivity/resistance to storage stress. In addition, the expression and activity of antioxidative enzymes were found to be correlated with sensitivity/resistance to pitting especially in the USA data. Higher antioxidative levels in the US in comparison to Israeli cherries might explain the lower pitting observed in California cherries. Moreover, an increase in antioxidative-related enzymes in Lapins Israel was observed only in the year with lower pitting. Interestingly, a bioinformatics ROSMETER analysis (determines the oxidative-related transcriptome footprint) on the Israeli data revealed a very strong ROS-related transcriptomic footprint only in the year of high pitting levels. Our results strongly suggest that pitting is related to oxidative stress.

Immersion of cherries in calcium alone (California) or with ethanol (Israel) resulted in inconsistent minor improvement of pit development. It is possible that this is because application of external calcium only slightly increased the total calcium or the apoplastic calcium of the fruit (California). Our results are similar to those found in the literature for apple fruit. There is a need to try different approaches to increase the calcium in the fruit. One such approach could be to improve calcium movement to the fruit by diverting calcium from the leaves to the fruits during fruit development.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Submitted	1	0	0

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
M.Sc. Student	Kolomos	yelena	Volcani Center	Israel
M.Sc. Student	Parra	Ana	Volcani Center	Israel
Postdoctoral Fellow	Richmond	Kelly	UC Davis	USA

Describe how the contribution of the collaboration between the laboratories contributed to the research

The collaboration enabled the Israeli team to determine the apoplastic calcium levels in different cherry developmental stages. This yielded information necessary to connect pitting to apoplastic calcium. The group in Israel aided in the bioinformatics analysis of the USA team. A comparison between transcriptomes of fruits from two different countries revealed that there are major differences in the transcriptome between the two countries even for the same cultivar. This led to a comparison of natural pitting in the two countries by sharing the more severe scale of the Israeli team. The pitting analysis revealed that cherries in California are less susceptible to pitting than in Israel. This comparison could not have been done without the collaboration between the two teams. Future study will try to improve the Israeli cherries based on knowledge from California.

Explain in lay terms the major achievements accomplished in the project today

Pitting which occurs during storage in cherry reduces the fruit value and any reduction in pitting can improve the farmer's income and customer satisfaction. In this study we attempted to understand the processes that lead to increased pitting and to develop methodologies to curtail this phenomenon. Our findings revealed that mature fruit were more sensitive to pit development than less mature fruit and hence, storage of less mature fruit at 4C will reduce pit development. The levels of apoplastic (space between the cells) calcium was lower in mature fruit in comparison to younger fruit. It is possible that lower calcium in the apoplast in the more mature fruit reduced the acclimation response and the increase in pit formation. Acclimation response is partially manifested by higher induction of a few genes encoding antioxidative enzymes which overcome the stress of storage. It is very difficult to increase the calcium levels in the cherry fruit by external application and different means of improving calcium in the fruit should be developed.

List any changes made to the original research plan

Our original plan was based on a protocol to induce pitting by calibrated impact developed previously in our labs, and also suggested in the literature. According to the literature, we assumed that this protocol will enable us to differentiate between sensitive and resistant cultivars. Hence, our suggested strategy was to follow events which occur after impact and storage. We hypothesized that early events in resistant cultivars following impact and exposure to cold (also desiccation which occurs in cold storage) are related to acclimation. Hence, we planned to investigate the physiological and molecular events occurring in resistant compared to sensitive cultivars during storage following an impact. We also suggested to follow events which occur during development of natural pitting without an impact. We planned to compare between impacted and non-impacted fruits in order to determine the events which are relevant to the impact. The physiological and molecular events we planned to follow during storage were: transcriptome analysis, calcium distribution in apoplast and the related esterified pectin moieties (more de-esterified moieties more cell wall bound calcium and less apoplastic calcium), ion leakage, ROS generation, oxidative-related luminescence, malondialdehyde (MDA), membrane fluidity, and cell viability. Special emphasis in the transcriptome analysis was supposed to be given to genes known to participate in acclimation as mentioned in the literature.

Since we observed that dipping cherries in ethanol and calcium reduced pit formation, we suggested to use this system as a functional analysis tool to enable studying the relevance of physiological and molecular change to pit formation. We hoped that determination of the mode of action of calcium and ethanol would also lead to practical treatments to reduce pit formation based on possible synergistic effects of these compounds.

There were several changes in our original plan which resulted from our initial observations in the first and second years of the project:

a. We could not confirm that the protocol of pitting induction by controlled force was suitable to differentiate between sensitive and resistant cultivars as seen in commerce. Hence, in Israel, we abandoned this system, but in the USA we added the system of natural pitting evaluation.
b. The variabilities in pitting between years and orchards made it very difficult, to compare sensitive and resistant cultivars. Nevertheless, we confirmed that Bing is a resistant cultivar in comparison to others, especially Lapins and Brooks, in several years.

c. We turned the pitfall of variabilities between years into an advantage and at least in Israel, we compared the same cultivar (Lapins) in two years with different pitting results.

d. Comparing the same cultivar with different pitting resulting from different conditions yielded the most information. For example, we determined that 0C storage caused more pitting than 4C in the same cultivar. We also showed that early mature fruit were more resistant than late mature fruit of the same cultivar. We were also able to show correlations between ROS-related genes and pitting between two years of the same cultivar.

e. We followed most of the parameters we proposed. However, the following parameters did not yield differences that could explain pitting. The parameters of ROS generation, membrane fluidity, and cell viability proved to be inconsistent and hence were abandoned. However, we did find differences in antioxidative enzymes gene expression and activity that potentially explain the differences in pitting.

f. We were not able to find differences between resistant and sensitive cultivars in the esterified pectin moieties due to Pectin Methyl Esterase (PME) activity, hence this line of research was abandoned.

g. We did not pursue the calcium and ethanol treatments since the results were not consistent.

Publications for Project IS-4858-15

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Submitted	Other	*1. Kochanek, BT., Naschitz, S., Para AS., Gamrasni, D., Friedman, H.*	The effect of the physiological age on pit development during storage in cherry	***Alon Hanotea***	In Press : 2019	IS only

Appendix of Scientific report 4858-15

Any data or publications you wish to add to the proposal

1. Summary of major scientific achievements
2. Figures supporting the statements in the document

Summary of major scientific achievements

Pitting in cherry, the collapse of the fruit surface, develops during storage in perfect fruit. In general, the severity of pitting in Israel cherries was higher than in California cherries. Following our initial study which suggest that fruit maturity might affect pitting, we examined pitting in two developmental stages for all cultivars. Harvested cherry were separated to two maturity stages based on their chroma, and it was found that fruit of a more mature stage exhibited higher pitting than the less advanced stage especially at 4C. Pitting develops after harvest even at 20C, however in most of our storage experiments, exposure to 0C induced a more severe pitting than exposure to 4-5C. Hence, it seems that 0C storage causes an additional stress, augmenting the tissue collapse. However, although this phenomenon is augmented at 0C, it is different from chilling stress of sensitive fruits like mango, avocado or tomato, where younger fruits are more sensitive than mature fruit to chilling. This observation is supported by the finding that although the pitting was more severe in 0C than at 4-5C, ion leakage was not higher at 0C, as seen in other crops.

Only in one year we could find a negative correlation between apoplastic calcium and induced pitting among the different cultivars (in following years no difference was found in induced pitting between cultivars). Moreover, we could not find a correlation between natural pitting and calcium (total and apoplastic). It is possible that the variations in pitting sensitivity between years and locations masked this phenomenon. Analysis of apoplastic calcium in the two developmental stages in harvest, revealed that the more mature stage had lower apoplastic calcium than less mature fruit. This observation may suggest that indeed lower apoplastic calcium is related to higher sensitivity to pitting development in more advanced stage. In conclusion, the role of low apoplastic calcium in pitting development has not been established yet.

Impact, either by dropping the fruit or by applying localized force, increased or caused pitting, respectively. However pitting is not caused only by an impact. This conclusion is based on the following observations: a. Pitting can develop in perfect fruit; b. Fruit can recover from a localized force. Hence, the tissue is equipped with mechanisms to overcome the impact; c. Cultivars differ in their pitting severity, however the severity is different between years and locations as well, indicating that multiple factors affect pitting, not only impact.

Application of localized controlled impact was suggested as a tool to differentiate between cultivars of different sensitivity to pitting, and a tool to investigate the course of events which lead to pitting. We showed that ROS and cell death increased under the pit. However, we could not find differences in reactive oxygen species or apoplastic calcium after induced pitting between the cultivars. Since induced pitting did not always reflect the sensitivity of natural pitting after storage (both in Israel and in the USA), we concluded that this method cannot be used as a phenotypic tool to predict differences in sensitivity to pitting between cultivars or years, despite publications in the literature. Nevertheless, we confirmed that generally, Lapins (Israel, California) and Brooks (California) are more sensitive than Bing (Israel, California) to the development of natural pitting.

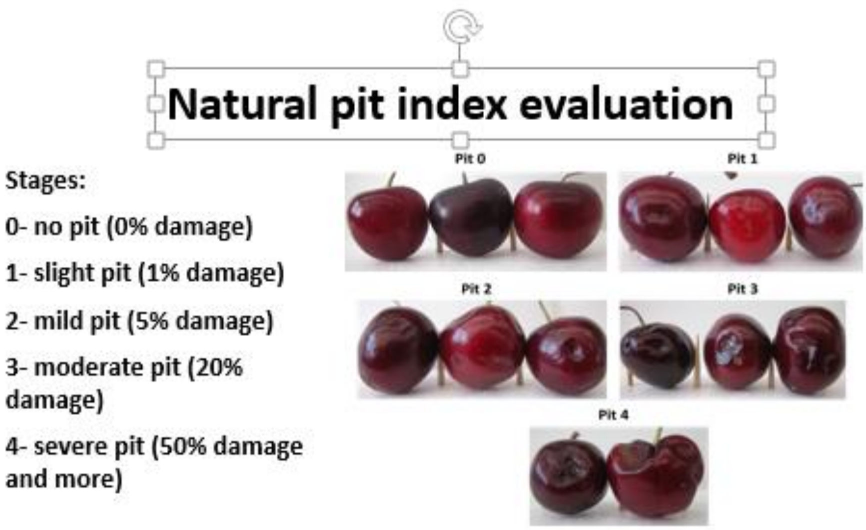
Transcriptome analysis of early stored cherries was performed on Bing and Lapins in two consecutive years in Israel and on impacted and chilled Bing and Brooks fruit in California in one year. There was a major difference in the transcriptome of cherries from California and from Israel. Correlation transcriptome matrix analysis revealed that the transcriptome of California Bing and Brooks is very different, but the changes during storage for each cultivar are very small. On the other hand, there were major changes during one to three days of storage in comparison to harvest in the cherries from Israel. The changes which occurred during storage in Israel, but less so in the USA, might explain the higher sensitivity to pitting of the Israeli cherries.

Correlation transcriptome matrix of Israeli fruit (Bing and Lapins) in two consecutive years revealed several distinct groups: a group which clustered all Bing and Lapins of day 0 of two years together, a group which include Bing storage in 2016 and 2017 and Lapins in 2017, and another group of Lapins storage in 2016. This indicates that most of the differences exist in transcriptome of Lapins storage between the 2016 and 2017. This difference between the two years correlated with differences in pitting in Lapins between the two years. More specifically, we examined the expression of genes within the cold acclimation pathway and found a major induction of *C-repeat binding factor (CBF)* and *ABA-Stress-Ripening* (*ASR3)* (major transcription factors involved in cold protection) in the USA transcriptome during storage, while major induction of *Sucrose Nonfermenting Related Kinases 2.6* (*SnRK2)* and *Inducer of CBF Expression* (*ICE)* (a kinase and transcription factor acting upstream of CBF, respectively) occurred in the Israeli cherry. The meaning of this result is still not clear.

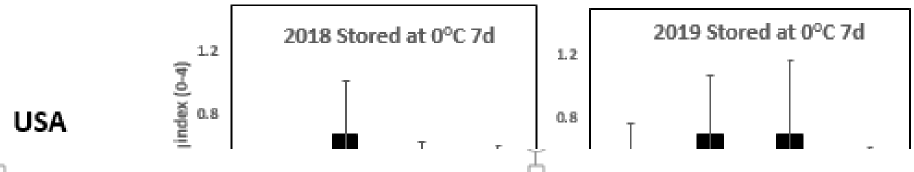
Mechanisms of acclimation to stress usually involve the induction of genes with antioxidative activity. *Glutathione Peroxidase* (*GPX), Glutathione Reductase* (*GR)* and *Alternative Oxidase* (*AOX)* were induced by cold mainly in 2017 in Lapins, Israel, fitting with low pitting in this year in this cultivar. On the other hand, no antioxidative activity was induced in 2016 when Lapins fruit developed high levels of pitting. Interestingly, when a ROSMETER analysis (analysis which enables us to determine the ROS-related transcriptome footprint) was performed on the transcriptome data from Israel, we found a very strong ROS-related transcriptomic footprint only in the year (2016) when fruit developed high levels of pitting. Brooks in the USA had lower expression of *AOX, Superoxide Dismutase* (*SOD)* and peroxidases than Bing at harvest and peroxidases were induced by cold mainly in Brooks. In addition, the enzyme activity of SOD and peroxidases were lower in Brooks than in Bing, suggesting that Brooks in the USA is not equipped with antioxidative capacity for the acclimation, most likely because of lower *CBF* expression than Bing at harvest.

Immersion of cherries in calcium alone (California) or with ethanol (Israel) resulted in inconsistent minor improvement of pit development. It is possible that this is because application of external calcium only slightly increased the total calcium or the apoplastic calcium of the fruit (California). Our results are similar to those found in the literature for apple fruit. There is a need to try different approaches to improve the calcium in the fruit. One such approach could be to improve calcium movement to the fruit by diverting calcium from the leaves to the fruits during fruit development.

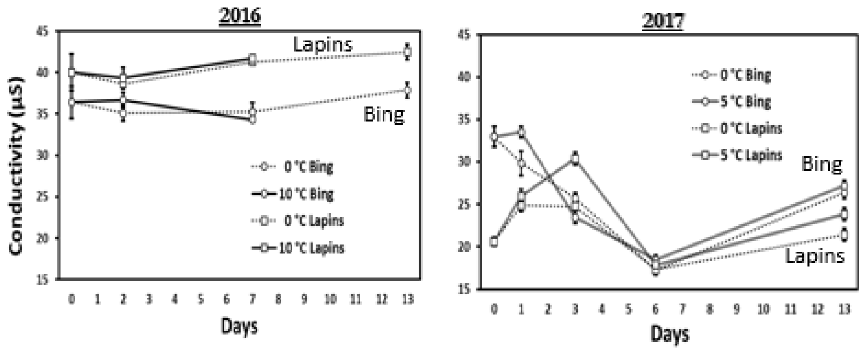
Figures supporting the major achievements



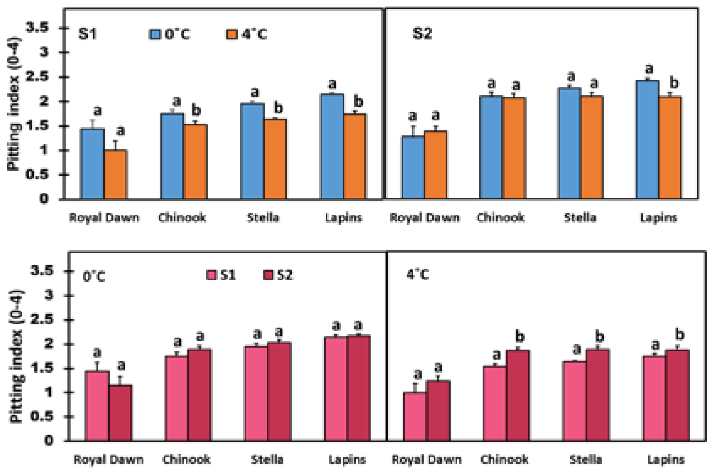
Severity of pitting in Israel is higher than in California



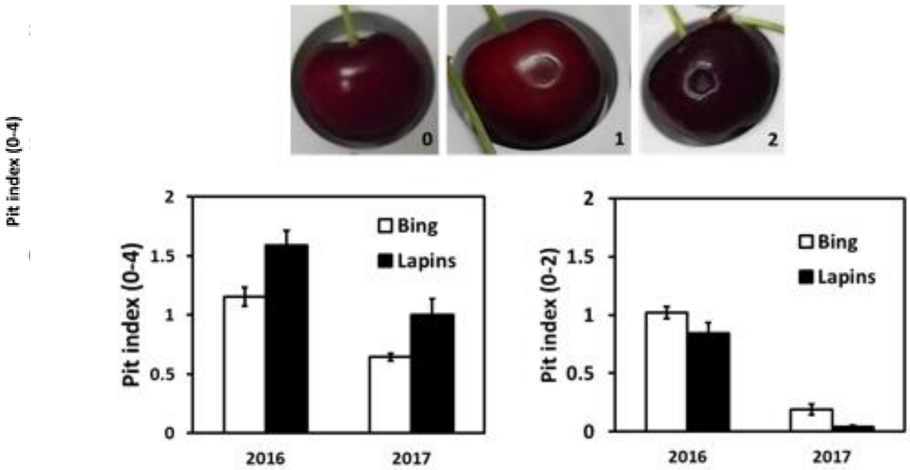
Ion leakage was not higher at 0C than in 5C although pitting was usually higher



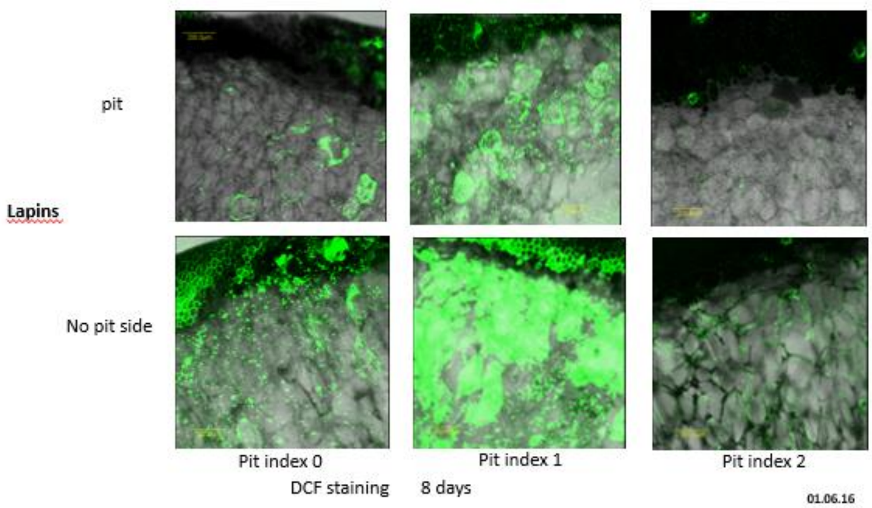
Storage at 0C increased pitting.
Stage 2 of maturity is more sensitive (2018 results, Israel).
Similar results were observed in 2019

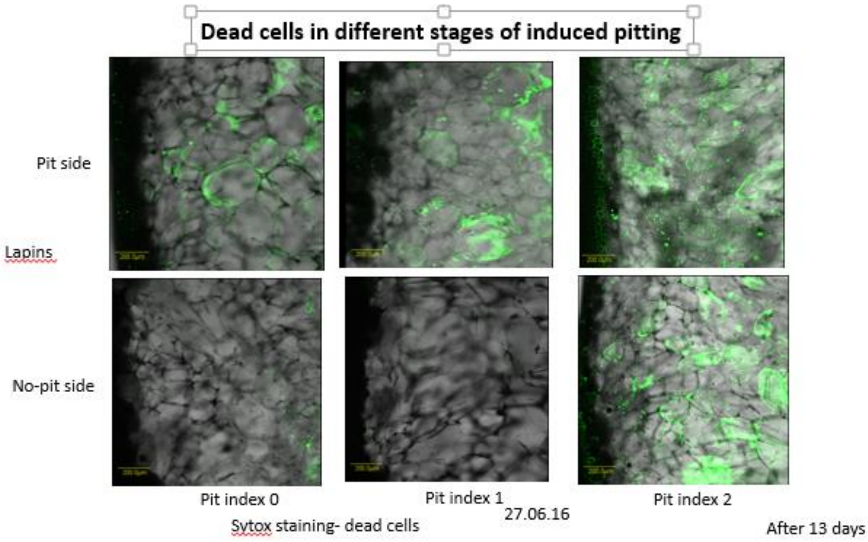


Induced pitting is not a good tool to differentiate between the sensitivity to natural pitting natural pitting

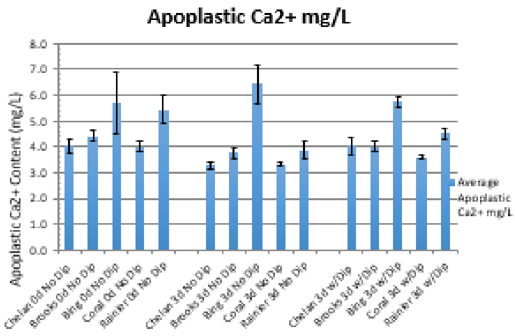


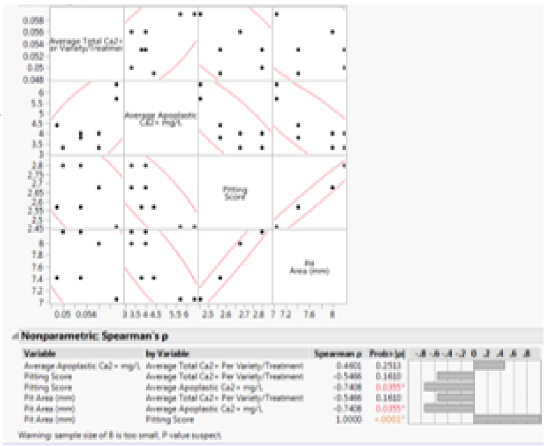
ROS staining in different stages of induced pitting

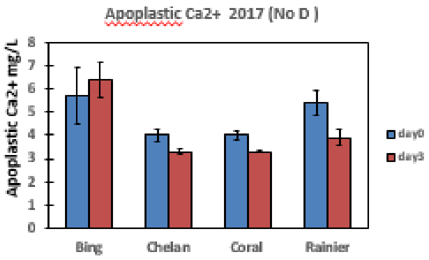



Dead cells in different stages of induced pitting
Pit side
Lapins
No-pit side
Pit index 0
Pit index 1
Pit index 2
27.06.16
Sytox staining- dead cells
After 13 days

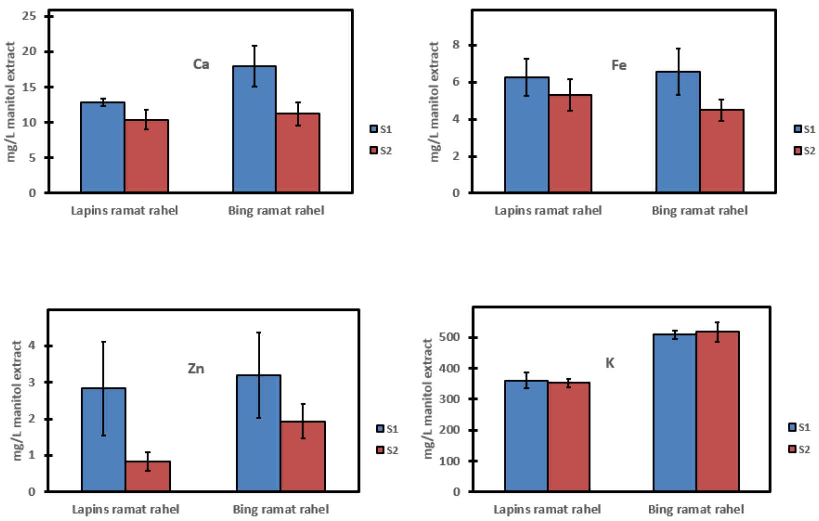
In 2017 (USA) negative correlation between induced pitting and apoplastic calcium



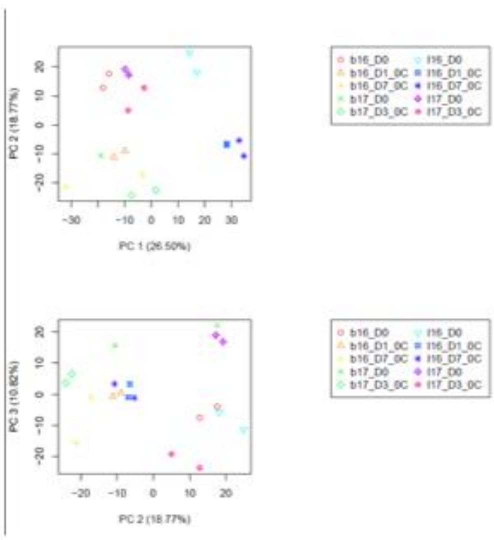


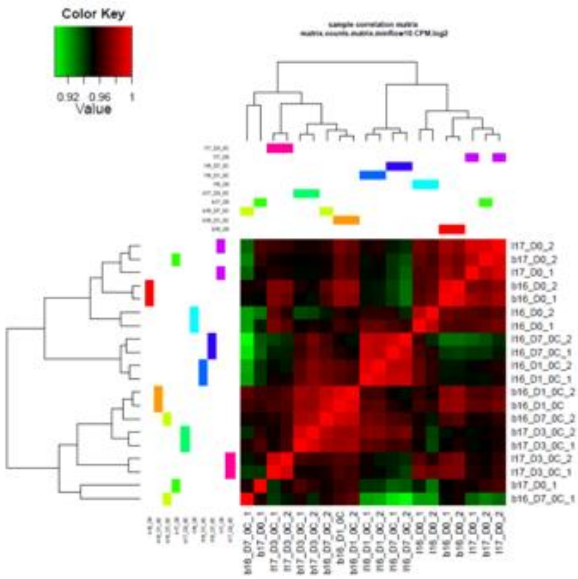


Cherries in advanced developmental stage contain in the apoplast less Ca, Fe and Zn, but not K

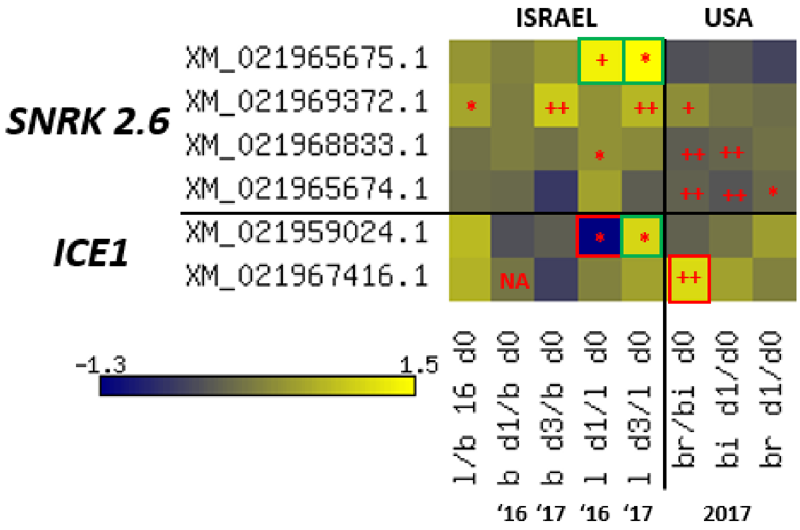


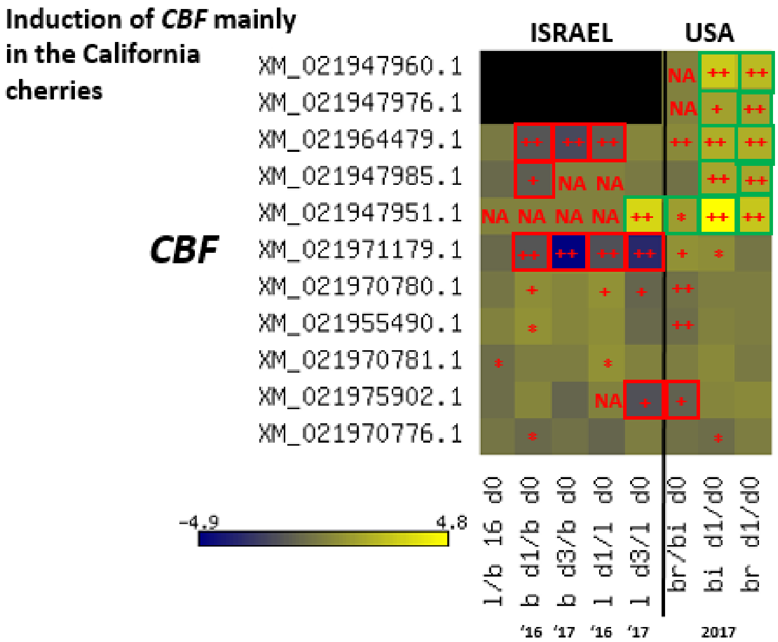
PCA and matrix analysis of Israel transcriptome revealed major changes between day 0 and storage but less between cultivar



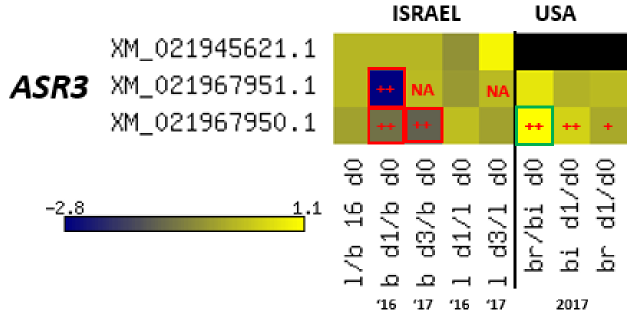


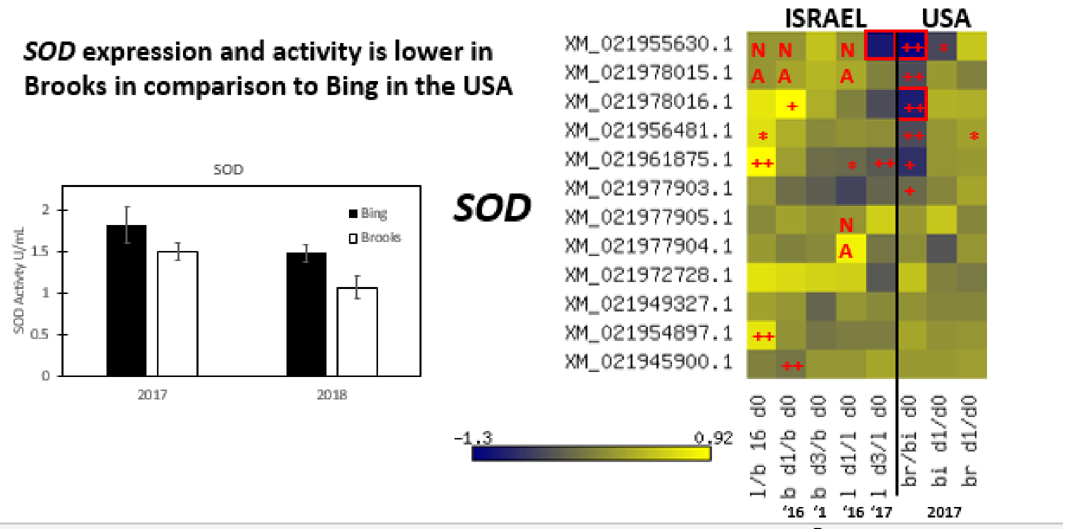
Induction of *SNRK2.6* and *ICE* in Lapins mainly in 2017

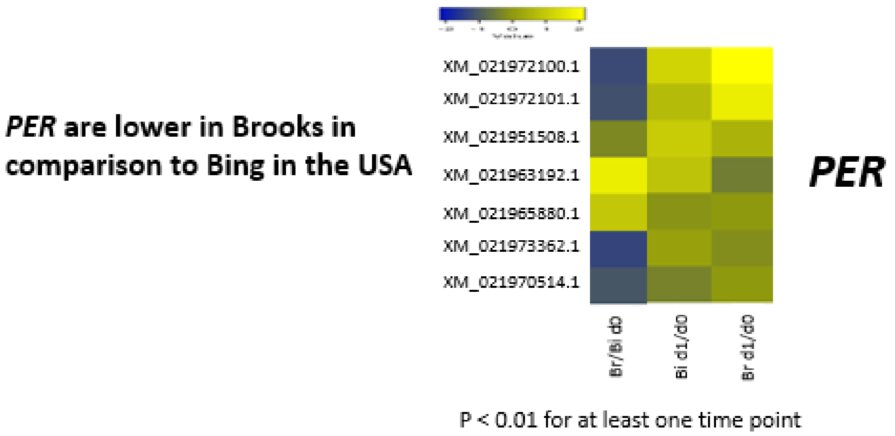




High *ASR3* in Brooks and reduction in Israel mainly in Bing Israel during storage

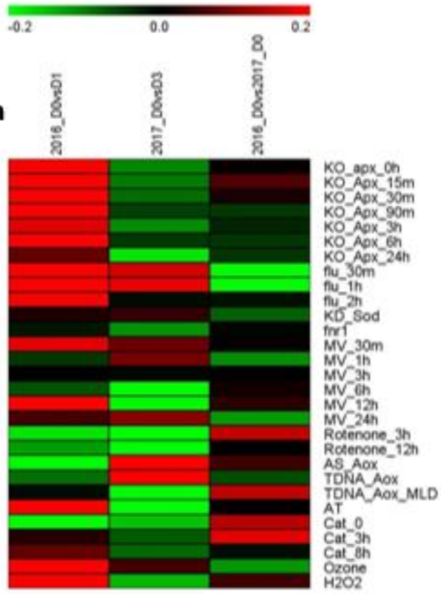



SOD expression and activity is lower in Brooks in comparison to Bing in the USA
SOD
SOD Activity U/mL
Bing
Brooks
2017
2018
SOD
ISRAEL
USA
XM_021955630.1
XM_021978015.1
XM_021978016.1
XM_021956481.1
XM_021961875.1
XM_021977903.1
XM_021977905.1
XM_021977904.1
XM_021972728.1
XM_021949327.1
XM_021954897.1
XM_021945900.1
-1.3
0.92
2017


PER are lower in Brooks in comparison to Bing in the USA
XM_021972100.1
XM_021972101.1
XM_021951508.1
XM_021963192.1
XM_021965880.1
XM_021973362.1
XM_021970514.1
Br/Bi d0
Bi d1/d0
Br d1/d0
PER
P < 0.01 for at least one time point

ROSMETER analysis of the transcriptome of Lapins from 2016 and 2017

Analysis revealed high correlation between oxidative stresses the change in transcriptome only for 2016 year when Lapins exhibited high levels of pitting



Application of external calcium only slightly increased the total calcium of the fruit.